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(54) Title: METHOD OF DETECTING THROMBOTIC DISEASE RISK

(57) Abstract

Polymorphisms within a plasma carboxypeptidase designated plasma carboxypeptidase B (PCPB) have been identified. The relative distribution of these polymorphs in a patient's blood can be used to assess an indivudual's risk toward thrombotic disease.

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METHOD OF DETECTING THROMBOTIC DISEASE RISK

Field of the Invention

This invention relates to a carboxypeptidase that binds plasminogen. In particular, it relates to a plasma carboxypeptidase designated plasma carboxypeptidase B (PCPB) and to the use of polymorphism in the gene for this enzyme to diagnose patients at risk for thrombotic disease.

BACKGROUND OF THE INVENTION

The coagulation and fibrinolytic cascades comprise a series of zymogen to enzyme conversions which terminate in the proteolytic enzymes thrombin and plasmin, respectively (Mann *et al.*, *Ann. N.Y. Acad. Sci.* (1991), Vol. 614, pp. 63-75); K. Collen *et al.*, *Blood* (1991), Vol. 78, pp. 3114-3124; Astrup T., *Semin. Thromb. Hemostasis* (1991), Vol. 17, pp. 161-174). These enzymes catalyze the deposition and removal of fibrin. A proper balance between the activities of the two cascades is required both to protect the organism from excessive blood loss upon injury and to maintain blood fluidity within the vascular system. Imbalances are characterized by either bleeding or thrombotic tendencies, the latter of which are manifested as, for example, heart attacks and strokes.

Thrombomodulin is a component of the blood vessel wall which binds thrombin and changes its specificity from fibrinogen to protein C, yielding anticoagulant rather than procoagulant activity (Esmon, C.T., *FASEB J.* (1995), Vol. 9, pp. 946-955). The thrombin-thrombomodulin complex catalyzes cleavage of protein C to activated protein C, which then downregulates the coagulation cascade by proteolytically inactivating the essential cofactors Factor Va and Factor VIIIa (Esmon *et al.*, *Ann. N. Y. Acad. Sci.* (1991), Vol. 614, pp. 30-43). These events are essential in the regulation of the coagulation cascade.

Early studies suggested that activated protein C is not only an anticoagulant but also profibrinolytic, both *in vitro* and *in vivo* (Taylor *et al.*, *Thromb. Res.* (1985), Vol. 37, pp. 639-649; de Fouw *et al.*, *Adv. Exp. Med. Biol.*, Vol. 281, pp. 235-243). It was later determined that protein C only appears profibrinolytic because it prevents the thrombin-catalyzed activation of a previously unknown fibrinolysis inhibitor, whose precursor has been isolated from plasma and designated TAFI (thrombin-activatable fibrinolysis inhibitor) or PCPB (plasma carboxypeptidase

B). The zymogen precursor is activated by thrombin, plasmin or by a thrombin-thrombomodulin complex to produce an enzyme with carboxypeptidase B activity, which inhibits plasminogen activation and thereby prolongs fibrinolysis (Bajzar *et al.*, *J. Bio. Chem.* (1996), Vol. 270, pp. 14477-14484).

TAFI was discovered independently in three different laboratories. It initially appeared as an unstable carboxypeptidase B-like entity in human serum and was described by Hendricks *et al.* (*Biochim. Biophys. Acta* (1990), Vol. 1034, pp. 86-92). Then Eaton *et al.* (*J. Biol. Chem.* (1991), Vol. 266, pp. 21833-21838) cloned the cDNA, deduced the amino acid sequence, described its activation by trypsin, and analyzed its enzymatic properties toward synthetic carboxypeptidase B substrates. They designated the protein PCPB, for plasma carboxypeptidase B (see U.S. Patent 5,206,161). Wang *et al.* (*J. Biol. Chem.* (1994), Vol. 269, pp. 15937-15944) independently isolated the activated material and named it carboxypeptidase U, where "U" indicates unstable. Nesheim *et al.* (*J. Biol. Chem.* (1995), Vol. 270, pp. 14477-14484) showed that the protein was both activated by thrombin and inhibits fibrinolysis and gave it the name TAFI (thrombin-activatable fibrinolysis inhibitor). Subsequently, Tan and Eaton (*Biochemistry* (1995), Vol. 34, pp. 5811-5816) studied the trypsin activated enzyme and renamed the protein plasma procarboxypeptidase B (pro-pCPB). The co-identity of TAFI and pro-pCPB (or PCPB, as it was initially designated) has been established by their chromatographic behavior on plasminogen Sepharose and the amino acid sequences present at the activation cleavage site.

Thrombophilia can be defined as a tendency toward venous thromboembolic disease in adults under 50 years old in the absence of known risk factors including, among others, malignancy, immobilization, or major surgery. In principle, a tendency toward venous thrombosis could arise from hyperactive coagulation pathways, hypoactive anticoagulant mechanisms, or hypoactive fibrinolysis. Molecular explanations for some thrombophilic patients have come following the discoveries of hereditary thrombophilia associated with deficiencies of the anticoagulant factors antithrombin III (Egeberg, O., *Throm. Diath. Haemorrh.* (1965) Vol. 13, p. 516), protein C (Griffin *et al.*, *J. Clin. Invest.* (1981), Vol. 68, p. 1370), and protein S (Comp *et al.*, *N. Engl. J. Med.* (1984), Vol. 31, p. 1525). More recently, Dahlback et al. (*Proc. Natl. Acad. Sci USA* (1993), Vol. 90, p.1004) have identified the presence of a single point mutation in the Factor V gene, which results in the replacement of an amino acid within the activated protein C cleavage site of the Factor Va molecule. The presence of this mutation has been useful in screening the population to determine those at risk for thromboembolic (thrombotic) disease.

SUMMARY OF THE INVENTION

The present invention arises from the discovery of the presence of two naturally occurring polymorphs of the PCPB protein, which contain different amino acids at position 147: $PCPB_{Ala147}$ and $PCPB_{Thr147}$.

Accordingly, the invention is directed to a process for determining the presence of DNA or protein polymorphs of PCPB in human subjects, said process comprising:

obtaining a tissue or blood sample from the subject;

preparing the sample for analysis; and

determining the presence of PCPB polymorphs within the sample.

The invention is further directed to a process for determining the presence of the genes coding for the $PCPB_{Ala147}$ and/or $PCPB_{Thr147}$ polymorphs in a human subject, comprising:

obtaining a blood sample from the subject;

Isolating genomic DNA from the blood sample;

amplifying segments of the genomic DNA associated with the PCPB gene using PCR;

separating the products of PCR using gel electrophoresis;

immobilizing the gel-separated products by transfer to a nylon membrane;

contacting the membrane with PCPB -specific probes; and

measuring the amount of hybridization of the probes with the membrane-immobilized DNA.

Another aspect of the invention is directed toward a process for determining the risk of thrombotic disease in a human subject, comprising comparing the relative distribution of $PCPB_{Ala147}$ and $PCPB_{Thr147}$ polymorphs within the subject with an at-risk population profile.

A further aspect of the invention is directed toward a kit for identifying human subjects at risk for thrombotic disease, comprising DNA probes useful in measuring polymorphisms within the PCPB gene of the subject and a table useful for comparing the subject's PCPB polymorph profile with an at-risk population profile.

Brief Description of the Drawings

Figure 1 is the nucleotide sequence of the human PCPB ($PCPB_{Thr147}$) (SEQ ID NO: 1) disclosed in *Eaton et al., J. Biol. Chem.* (1991), Vol. 266, pp. 21833-21838, with the positions of the nucleic acid substitutions (505 and 678) found in the newly isolated polymorph underlined.

In the polymorph designated $PCPB_{Ala147}$, the following substitutions have occurred: 505 (A to G) and 678 (C to T).

Figure 2 provides an amino acid sequence of the PCPB protein (SEQ ID NO: 2) produced from the nucleic acid sequence shown in Figure 1, in which the amino acid at position 147 isThr. In $PCPB_{Ala147}$, the amino acid at position 147 is Ala.

Figure 3 illustrates carboxypeptidase B activity of isolated activated recombinant $PCPB_{Thr147}$ and $PCPB_{Ala147}$.

Figure 4A shows Southern blot analysis of DNA from plasmids containing either $PCPB_{Thr147}$ or $PCPB_{Ala147}$ cDNA, using DNA probes specific for each polymorph. Figure 4B shows Southern blot analysis of amplified genomic DNA isolated from a human blood specimen, using the same probes.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

As used in the specification and appended claims, unless specified to the contrary, the following terms have the meaning indicated:

The term "PCPB" refers to the protein described by Eaton *et al.* (*J. Biol. Chem.* (1991), Vol. 266, pp. 21833-21838 and which has an amino acid sequence substantially homologous to that shown in Figure 2 (SEQ ID NO: 2).

The term "$PCPB_{Thr147}$ " refers to an isolated polypeptide whose sequence was published in Eaton *et al.* (*J. Biol. Chem.* (1991), Vol. 266, pp. 21833-21838) and whose amino acid sequence is shown in Figure 2 (SEQ ID NO: 2).

The term "$PCPB_{Ala147}$ " refers to an isolated polypeptide which has an amino acid sequence identical to that of $PCPB_{Thr147}$, except for the substitution of the amino acid Thr by Ala at position 147.

A polypeptide "fragment" or "segment" refers to a stretch of amino acid residues of at least about 6 contiguous amino acids from a particular sequence, more typically at least about 12 amino acids but can be up to 20 amino acids.

A "fragment" or "segment" of a nucleic acid refers to a stretch of at least about 18 nucleotides, more typically at least about 50 to 200 nucleotides but less than 2 kb.

A "polymorphism" refers to a genetically determined heterogeneity of proteins, especially enzymes, and tend to occur when the frequency of a genetic variant in a population is greater than 1%. Frequencies of this order develop by positive selection or by the effect of incidental

genetic drift on rare mutations that have a heterozygotic advantage. The resulting polymorphs of a protein differ from each other by substitution or deletion of an amino acid at one or more sites in the peptide chain.

A "polymorph" in the context of a nucleic acid or a gene is an alternative form (allele) of the gene that exists in more than one form in the population. At the polypeptide level, "polymorphs" generally differ from one another by only one, or at most, a few amino acid substitutions.

The term "recombinant" or "recombinant DNA molecule" refers to a polynucleotide sequence which is not naturally occurring, or is made by the artificial combination of two otherwise separated segments of sequence. By "recombinantly produced" is meant artificial combination often accomplished by either chemical synthesis means, or by the artificial manipulation of isolated segments of nucleic acids, e.g., by genetic engineering techniques. Such is usually done to replace a codon with a redundant codon encoding the same or a conservative amino acid, while typically introducing or removing a sequence recognition site. Alternatively, it is performed to join together nucleic acid segments of desired functions to generate a single genetic entity comprising a desired combination of functions not found in the common natural forms. Restriction enzyme recognition sites, regulation sequences, control sequences, or other useful features may be incorporated by design. "Recombinant DNA molecules" include cloning and expression vectors.

The terms "isolated", "substantially pure", and "substantially homogenous", are used interchangably and describe PCPB protein or polypeptide, or fragments thereof, or a DNA segment encoding same, where such protein or peptide, or DNA molecule is separated from components that naturally accompany it. An PCPB polypeptide or fragment thereof, or DNA segment encoding same is substantially free of naturally-associated components when it is separated from the native contaminants which accompany it in its natural state. Thus, a polypeptide that is chemically synthesized or synthesized in a cellular system different from the cell in which it naturally originates will be sustantially free from its naturally-associated components. Similarly, a nucleic acid that is chemically synthesized or synthesized in a cellular system different from the cell in which it naturally originated will be substantially free from its naturally-associated components.

The term "homologous", when used to describe a nucleic acid, indicates that two nucleic acids, or designated sequences thereof, when optimally aligned and compared, are identical, with appropriate nucleotide insertions or deletions, in at least 60% of the nucleotides, usually from

about 75% to 99%, and more preferably at least about 98 to 99% of the nucleotides.

The term "polymerase chain reaction" or "PCR" refers to a procedure wherein specific pieces of DNA are amplified as described in U.S. Pat. No. 4,683,195, issued 28 Jul. 1987. Generally, sequence information from the ends of the polypeptide fragment of interest or beyond needs to be available, such that oligonucleotide primers can be designed; these primers will point towards one another, and will be identical or similar in sequence to opposite strands of the template to be amplified. The 5' terminal nucleotides of the two primers will coincide with the ends of the amplified material. PCR can be used to amplify specific DNA sequences from total genomic DNA, cDNA transcribed from total cellular RNA, plasmid sequences, etc. See generally Mullis et al., *Cold Spring Harbor Symp. Quant. Biol.*, 51: 263 (1987); Erlich, ed., *PCR Technology*, (Stockton Press, NY, 1989).

The term "residue" refers to an amino acid that is incorporated into a peptide. The amino acid may be a naturally occurring amino acid and, unless otherwise limited, may encompass known analogs of natural amino acids that can function in a similar manner as naturally occurring amino acids. For purposes of this disclosure, amino acid residues are designated herein by their accepted three-letter or one-letter abbreviation, or by the notation "AA", which signifies the presence of an amino acid residue. The amino acids referred to herein are described by shorthand designations as follows:

Table 2: Amino Acid Nomenclature

Name	3-letter	1 letter
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamic Acid	Glu	E
Glutamine	Gln	Q
GlycineGly	G	
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K

Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

The terms "peptides" and "polypeptides" refer to chains of amino acids whose αcarbons are linked through peptide bonds formed by a condensation reaction between the α carbon carboxyl group of one amino acid and the amino group of another amino acid. The terminal amino acid at one end of the chain (amino terminus) therefore has a free amino group, while the terminal amino acid at the other end of the chain (carboxy terminus) has a free carboxyl group.

The term "amino terminus" (abbreviated N-terminus) refers to the free α-amino group on an amino acid at the amino terminal end of a peptide or to theα-amino group (imino group when participating in a peptide bond) of an amino acid at any other location within the peptide. Similarly, the term "carboxy terminus" (C-terminus) refers to the free carboxyl group on the carboxy terminal end of a peptide or the carbonyl group of an amino acid at any other location within the peptide. Typically, amino acids comprising a polypeptide are numbered in order, increasing from the amino terminus to the carboxy terminus of the polypeptide. Thus when one amino acid is said to "follow" another, that amino acid is positioned closer to thecarboxy terminal of the polypeptide than the "preceding" amino acid.

The term "immunoglobulin", "antibody" or "antibody peptide(s)" refers to polyclonal antibodies, monoclonal antibodies, to an entire immunoglobulin or antibody or any functional fragment of an immunoglobulin molecule which binds to the target antigen. Examples of such immunoglobulins include complete antibody molecules, antibody fragments, such as Fab, F(ab')2, complementarity determining regions (CDRs), VL (light chain variable region), VH (heavy chain variable region), and any combination of those or any other functional portion of an antibody peptide.

Much of the nomenclature and general laboratory procedures referred to in this application can be found in Sambrook et.al., *Molecular Cloning, A Laboratory Manual* (2nd Ed.),

Vol 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989 or in Berger and Kimmel, *Guide to Molecular Cloning Techniques, Methods in Enzymology* 152 (Academic Press, Inc., San Diego, CA). The manuals are hereinafter referred to as 'Sambrook" or "Berger" respectively, and are each incorporated herein by reference.

Isolation of PCPB cDNA

DNA encoding PCPB is obtained from a liver cDNA library, or genomic DNA, or by *in vitro* synthesis. Identification of PCPB DNA most conveniently is accomplished by probing human cDNA or genomic libraries with labelled oligonucleotide sequences selected from the sequence published in *Eaton et al.* in accord with known criteria, among which is that the sequence should be of sufficient length and sufficiently unambiguous that false positives are minimized. Typically, a ^{32}P-labelled oligonucleotide having 30 to 50 bases is sufficient, particularly if the oligonucleotide contains one or more codons from methionine or tryptophan. "Isolated" nucleic acid will be nucleic acid that is identified and separated from contaminant nucleic acid encoding other polypeptides from the source of nucleic acid. In the preferred embodiment, PCR was utilized to isolate cDNA clones coding for PCBP polymorphs as described in Examples 1 and 2.

Of particular interest is PCPB nucleic acid that encodes a full-length molecule, including but not necessarily the native signal sequence thereof. Nucleic acid encoding full-length protein is obtained by screening selected cDNA or genomic libraries using the amino acid sequence disclosed in *Eaton et al.*, and, if necessary, using conventional primer extension procedures to secure DNA that is complete at it 5' coding end. Such a clone is readily identified by the presence of a start codon in reading frame with the original sequence.

Cloning of PCPB

A variety of methods for cloning DNA sequences into prokaryotic cells are well known in the art. Organisms which are commonly utilized as hosts for the amplification of a vector include *Escherichia, Bacillus and Streptomyces.* The most common bacterial hosts are various commercially available strains of *E. coli*, due to the ease with which the organism may be cultured and the wealth of information which is available regarding the cell's life-cycle, genetics, viruses and developmental regulation. The vectors most commonly used in *E. coli* are those derived from the pBR322 plasmid and those derived from lambda or M13 phage, although several vectors unrelated to any of these are also common. The Sambrook and Berger manuals

contain methodology sufficient to direct persons of skill through most cloning exercises.

A number of vectors detailed in Sambrook and elsewhere may be initially cloned into *E. coli* and then subsequently transferred into a eukaryotic system without any necessity for re-cloning that part of the vector which is of interest to the person of skill. Vectors capable of replication in both prokaryotic and eukaryotic cells are generally termed "shuttle vectors" and must contain at a minimum a eukaryotic and a prokaryotic origin of replication. Several shuttle vectors are commercially available which contain multi-cloning sites, selectable markers for both bacterial and eukaryotic cells, promoters for both bacterial and eukaryotic expression of the gene(s) of interest, and integration sequences for insertion of the vector into the eukaryotic genome. A few examples of vectors which may be amplified in bacteria and used for transformation in eukaryotic cells include the family of P element vectors for *Drosophila melanogaster*, a number of SV40-derived vectors for the transformation of COS cells, adenovirus-derived vectors for transformation in cells containing the appropriate transcription factor for RNA polymerase III, a variety of BPV-derived vectors and the YIp5-derived vectors of *Sacchromyces cerevosiae* (see Sambrook chapter 16 and Berger chapter 53 for an overview of different vectors which may be transferred between *E. coli* and eukaryotes). General techniques for shuttling DNA between prokaryotes and eukaryotes are also described in Cashion et.al., U.S. Patent number 5,017,478 and Kriegler, *Gene Transfer and Expression: A Laboratory Manual*, W.H. Freeman, N.Y., (1990) which are incorporated by reference.

In the preferred embodiment, the mammalian expression vector pcDNA3 and the baculovirus expression vector pBacPAK8 were used.

Southern blot analysis of genomic DNA and northern blot analysis of RNA using a cloned probe are basic to the art of molecular biology. Sambrook provides adequate guidance to perform most commonly used southern and northern techniques including analysis of genomic DNA, mRNA and cDNA. The present invention provides an array of probes generated from the sequence of any region of the PCPB gene, probes generated from cleavage product of the cloned gene using random-primer or terminal phosphate labeling methods and several other methods known to persons of skill. The probes may be used for a variety of purposes including isolation of homologous genes from other species by screening genomic or expression libraries or performing PCR, identification of PCPB in tissues which express the PCPB gene using *in situ* or northern analysis, and identification of conditions which influence PCPB expression.

Expression of PCPB

Once the DNA encoding the PCPB protein is isolated and cloned, one may express the ligand in a recombinantly engineered cell such as bacteria, yeast, insect (especially employing baculoviral vectors), and mammalian cells. Methods for expression of recombinant proteins may be found in Sambrook chapters 16 and 17. It is expected that those of skill in the art are knowledgeable in the numerous expression systems available for expression of the DNA encoding PCPB protein. No attempt to describe in detail the various methods known for the expression of proteins in prokaryotes or eukaryotes will be made.

In brief summary, the expression of natural or synthetic nucleic acids encoding PCPB protein will typically be achieved by operably linking the DNA or cDNA to a promoter (which is either constitutive or inducible), and then incorporating the promoter-DNA construct into an expression vector. The vector should be suitable for replication and integration in either prokaryotes or eukaryotes. Typical expression vectors contain transcription and translation terminators, initiation sequences, and promoters useful for regulation of the expression of the polynucleotide sequence encoding PCPB protein. To obtain high level expression of a cloned gene, such as those polynucleotide sequences encoding PCPB protein, it is desirable to construct expression plasmids which contain, at the minimum, a strong promoter to direct transcription, a ribosome binding site for translational initiation, and a transcription/translation terminator.

a. Expression in Prokaryotes. Methods for the expression of cloned genes in bacteria are well known. Generally, to obtain high level expression of a cloned gene in a prokaryotic system, it is essential to construct expression vectors which contain, at the minimum, a strong promoter and regulator to direct mRNA transcription and termination. Examples of regulatory regions suitable for this purpose are the promoter and operator region of the *E. coli* β-galactosidase gene, the *E. coli* tryptophan biosynthetic pathway, or the leftward promoter from the phage lambda. The inclusion of selection markers in DNA vectors transformed in *E. coli* aid in the isolation of transformed bacteria. Examples of such markers include the genes specifying resistance to ampicillin, tetracycline, or chloramphenicol. In a preferred embodiment, a pUC19 - based vector was used for the subcloning and amplification of the desired gene sequences.

The PCPB protein produced by prokaryotic cells may not fold properly. During purification from *E. coli*, the expressed polypeptides may first be denatured and then renatured. This can be accomplished by solubilizing the bacterially produced proteins in a chaotropic agent such as guanidine HCl and reducing all cysteine residues with a reducing agent such as

beta-mercaptoethanol. The polypeptides are then renatured, either by slow dialysis or by gel filtration (see U.S. Patent No. 4,511,500).

b. Expression in Eukaryotes. A variety of eukaryotic expression systems such as yeast, insect cell lines and mammalian cells, are known to those of skill in the art. As explained briefly below, PCPB proteins may also be expressed in these eukaryotic systems.

1. Expression in Yeast. Synthesis of heterologous proteins in yeast is well known and described. *Methods in Yeast Genetics*, Sherman, F., *et al.*, Cold Spring Harbor Laboratory, (1982) is a well recognized work describing the various methods available to produce the AG175 polypeptides in yeast. Examples of promoters for use in yeast include GALI,IO (Johnson and Davies, *Mol. Cell. Biol.*, 4: 1440-1448 (1984)) ADH2 (Russell *et al., J. Biol. Chem.*, 258: 2674-2682 (1983)), PHO5 (*E.M.B.O.J.*, 6: 675-680, (1982)), and MFal (Herskowitz and Oshima, pp. 181-209 in *The Molecular Biology of the Yeast Saccharomyces*, Strathern *et al.*, eds. Cold Spring Harbor Lab., Cold Spring Harbor, N.Y. (1982)). A multicopy plasmid with a selective marker such as Leu-2, URA-3, Trp-I, and His-3 is also desirable.

A number of yeast expression plasmids like YEp6, YEpI3, YEp4 can be used as vectors. A gene of interest can be fused to any of the promoters in various yeast vectors. The above-mentioned plasmids have been fully described in the literature. See, for example, Botstein, *et al.*, *Gene*, 8:17-24 (1979) and Broach *et al.*, *Gene*, 8: 121-133 (1979).

Two procedures are used in transforming yeast cells. In one case, yeast cells are first converted into protoplasts using zymolyase, lyticase or glusulase, followed by addition of DNA and polyethylene glycol (PEG). The PEG-treated protoplasts are then regenerated in a 3% agar medium under selective conditions. Details of this procedure are given in the papers by Beggs *Nature*, 275: 104-109 (1978) and Hinnen, *et al. Proc. Natl. Acad. Sci. USA*, 75: 1929-1933 (1978). The second procedure does not involve removal of the cell wall. Instead the cells are treated with lithium chloride or acetate and PEG and put on selective plates. Ito *et al.*, *J. Bact.*, 153:163-168 (1983).

The PCPB protein can be isolated from yeast by lysing the cells and applying standard protein isolation techniques to the lysates. The monitoring of the purification process can be accomplished by using Western blot techniques or radioimmunoassays or other standard immunoassay techniques.

2. Expression in Mammalian and Insect Cell Cultures. The DNA sequences encoding PCPB proteins can be ligated to various expression vectors for use in transforming host cell cultures. The vectors preferably contain a marker such as dihydrofolate reductase or

metallothionein to provide a phenotypic trait for selection of transformed host cells. Cell cultures useful for the production of the PCPB protein are cells of insect or mammalian origin. Mammalian cell systems often will be in the form ofmonolayers of cells although mammalian cell suspensions may also be used. Illustrative examples of mammalian cell lines include VERO and HeLa cells, Chinese hamster ovary (CHO) cell lines, WI38, BHK, Cos-7 or MDCK cell lines. In preferred embodiments, CHO and BHK cells were used.

As indicated above, the vector, e.g., a plasmid, which is used to transform the host cell, preferably contains DNA sequences to initiate transcription and sequences to control the translation of the DNA sequence encoding the modified ligand. These sequences are referred to as expression control sequences. When the host cell is of insect or mammalian origin illustrative expression control sequences are obtained from the SV-40 promoter (*Science*, 222: 524-527 (1983)), the CMV I.E. promoter (*Proc. Natl. Acad. Sci.*, 81: 659-663 (1984)) or the metallothionein promoter (*Nature*, 296: 39-42 (1982)). The cloning vector containing the expression control sequences is cleaved using restriction enzymes and adjusted in size as necessary or desirable and ligated with polynucleotides coding for the PCPB protein by means well known in the art. In the preferred embodiment, either a mammalian expression vector, pcDNA3, was used with CHO or BHK cell or a baculovirus expression vector, pBacPAK8, was employed with Sf9 cell. A description of expression using the latter system is presented in Example 3.

As with yeast, when higher animal host cells are employed, polyadenlyation or transcription terminator sequences from known mammalian genes need to be incorporated into the vector. An example of a terminator sequence is the polyadenlyation sequence from the bovine growth hormone gene. Sequences for accurate splicing of the transcript may also be included. An example of a splicing sequence is the VP1 intron from SV40 (Sprague *et al.*, *J. Virol.*, 45: 773-781 (1983)).

Additionally, gene sequences to control replication in the host cell may be incorporated into the vector such as those found in bovine papilloma virus type-vectors. See, for example, Saveria-Campo, at pp. 2133-238 in *DNA Cloning Vol. II A Practical Approach*, D.M. Glover, ed. IRL Press, Arlington, VA (1985).

The host cells are competent or rendered competent for transformation by various means. There are several well-known methods of introducing DNA into animal cells. These include: calcium phosphate precipitation, fusion of the recipient cells with bacterial protoplasts containing the DNA, treatment of the recipient cells with liposomes containing the DNA, DEAE dextran, electroporation and micro-injection of the DNA directly into the cells.

The transformed cells are cultured by means well known in the art. See, for example, Kuchler *et al., Biochemical Methods in Cell Culture and Virology*, (1977). The expressed PCPB protein is isolated from cells grown as suspensions or as monolayers. They are recovered by well known mechanical, chemical or enzymatic means.

c. Expression in recombinant vaccinia virus- or adenovirus-infected cells. In addition to use in recombinant expression systems, the DNA encoding PCPB protein can also be used to transform viruses that transfect host cells *in vitro* or *in vivo*. These transfected host cells, in turn express the PCPB protein (see section on expression of PCPB proteins in eukaryotic cells, above).

Suitable viruses for use in the present invention include, but are not limited to, pox viruses, such as canarypox and cowpox viruses, and vaccinia viruses, alpha viruses, adenoviruses, and other animal viruses. The recombinant viruses can be produced by methods well known in the art, for example, using homologous recombination or ligating two plasmids. A recombinant canarypox or cowpox virus can be made, for example, by inserting the polynucleotides encoding the PCPB polypeptides into plasmids so that they are flanked by viral sequences on both sides. The polynucleotides encoding the PCPB polypeptide are then inserted into the virus genome through homologous recombination.

For example, a recombinant adenovirus can be produced by ligating together two plasmids each containing about 50% of the viral sequence and a nucleotide sequence encoding an PCPB polypeptide. Recombinant RNA viruses such as the alpha virus can be made via a cDNA intermediate using methods known in the art.

In the case of vaccinia virus (for example, strain WR), the nucleotide sequence encoding PCPB polypeptide can be inserted in the genome by a number of methods including homologous recombination using a transfer vector, pTKgpt-OFIS as described in Kaslow *et al., Science* 252:1310-1313 (1991), which is incorporated herein by reference.

Alternately the DNA encoding PCPB protein may be inserted into another plasmid designed for producing recombinant vaccinia, such as pGS62 (Langford *et al., Mol. Cell. Biol.* 6: 3191-3199 (1986)). This plasmid consists of a cloning site for insertion of foreign genes, the P7.5 promoter of vaccinia to direct synthesis of the inserted gene, and the vaccinia TK gene flanking both ends of the foreign gene.

Confirmation of production of recombinant virus can be achieved by DNA hybridization using cDNA encoding PCPB protein and by immunodetection techniques using antibodies specific for the expressed PCPB protein. Virus stocks may be prepared by infecting cells and

harvesting virus progeny.

Purification of PCPB Proteins

The PCPB proteins produced by recombinant DNA technology may be purified by standard techniques well known to those of skill in the art. Where the recombinant protein is secreted directly into the media the media is collected directly. Where the protein is retained either in solution within the cell or as an inclusion body, the cell must be lysed to recover the protein. This is typically accomplished by sonification or maceration.

In either case, the protein is then typically isolated from the cellular debris by filtration, centrifugation, or other means known to those of skill in the art, usually by filtration or centrifugation. The protein is then concentrated by adsorption to any suitable resin such as, for example, Q Sepharose or metal chelators, by ammonium sulfate fractionation, polyethylene glycol precipitation, dialysis, or by ultrafiltration. Other means known in the art may be equally suitable.

If the recombinant PCPB protein is expressed as a fusion protein, it may be necessary to digest the fusion protein with an appropriate proteolytic enzyme or use chemical cleavage (i.e. cyanogen bromide) to release the desired PCPB protein.

Purification of PCPB protein may require the additional use of, for example, gel electrophoresis, capillary electrophoresis, reverse phase HPLC, affinity chromatography, ion exchange chromatography, sizing chromatography or other protein purification techniques well known to those of skill in the art. See, for instance, Scopes, *Protein Purification: Principles and Practice*, Springer-Verlag: New York (1982), *Methods in Enzymology, Vol. 182: Guide to Protein Purification*. Deutscher, ed. Academic Press, Inc. N.Y. (1990) both of which are incorporated herein by reference.

In the preferred embodiment, recombinant PCPB proteins ($PCPB_{Ala147}$ and $PCPB_{Thr147}$) were each purified from media using S-sepharose chromatography and plasminogen-affinity chromatography as described in Example 4.

Antibody Production

Full length PCPB protein or fragments thereof will be useful for producing antibodies, either polyclonal or monoclonal. A multitude of techniques available to those skilled in the art for production and manipulation of various immunoglobulin molecules can be readily applied to

produce antibodies for use in the present invention. Antibodies which bind to PCPB protein may be produced by a variety of means. The production of non-human monoclonal antibodies, e.g., murine, lagomorpha, equine, etc., is well known and may be accomplished by, for example, immunizing the animal with a preparation of isolated PCPB molecules. Techniques for producing antibodies are well known in the literature, see, e.g., Goding, et al., *Monoclonal Antibodies: Principles and Practice* (2nd ed.) Academic Press, N.Y.; Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York (1988); and are exemplfied by U.S. Patent Nos 4,381,292, 4,451,570 and 4,618,577, which are each incorporated herein by reference. Antibody production against PCPB is exemplified in U.S. Patent No. 5,474,901, which is incorporated herein by reference.

The antibodies generated can be used for a number of purposes, e.g., as probes, in immunoassays, in diagnostics or therapeutics, or in basic studies seeking to dissect the portions of the protein responsible for the described properties of PCPB protein or fragments thereof.

Characterization of PCPB Polymorphs

The PCPB polymorphs are characterized in terms of i) their physicochemical properties, ii) *in vitro* activities and iii) *in vivo* activities. Studies of physicochemical properties include determination of molecular weight, carbohydrate content/sequence, isoelectric point, amino acid composition, amino acid sequence, and peptide mapping according to the general methods found in Hugli, ed., *Techniques in Protein Chemistry*, Academic Press, Inc. N.Y. (1989) and Deutscher et al., *Methods in Enzymology, Vol. 182: Guide to Protein Purification*, Academic Press, Inc., N.Y. (1990)), which are each incorporated herein by reference.

In vitro activities which can be examined are activation kinetics (binding affinity to thrombin and thrombomodulin), kinetics of carboxypeptidase B-like activity, stability in various media, binding affinity to plasminogen, as well as effects on *in vitro* clot lysis time. Briefly, PCPB polymorphs are activated by a thrombin/thrombomodulin complex, and effects of thrombin and thrombomodulin concentrations, incubation time and incubation temperature on the activation are studied by following carboxypeptidase B-like activity and the presence of the activation fragment by electrophoresis on SDS/PAGE gels.

Carboxypeptidase B-like activity is assayed by measuring the hydrolysis of substrates such as hippuryl-arginine (Folk *et al., J. Biol. Chem.* (1960), Vol. 235, pp. 2272-2277) or furylacryloyl-alanyl-lysine (Plummer and Kimmel, *Anal. Biochem.* (1980), Vol.108, pp. 348-353). The product, hippuric acid, may be converted to a chromogen to improve the sensitivity

of the assay (Hendriks *et al., Clinica Chimica Acta* (1986), Vol. 157, pp. 103- 108. The effects of PCPB polymorphs on clot lysis time are investigated using a general plate clot lysis assay (for instance, Beebe and Aronson, *Thrombosis Research* (1987), Vol. 47, pp. 123-128; Jones and Meunier, *Thrombosis and Haemostasis* (1990), Vol. 64, pp. 455-463; Bajzar *et al., J. Biol. Chem.* (1996), Vol. 271, pp. 16603-16608). Spontaneous fibrinolysis in whole human plasma may be studied (for example, as described by Wun and Capuano, *J. Biol. Chem.* (1985), Vol. 260, pp. 5061-5066).

Pharmacokinetics and pharmacology of the two polymorphs *in vivo* can also be examined. General pharmacokinetics such as clearance rate and plasma half-life are determined using techniques described in *Principles of Drug Action*, Goldstein *et al.*, ed., John Wiley and Sons (1973), which is incorporated herein by reference. The pharmacology of $PCPB_{Thr147}$ and $PCPB_{Ala147}$ may be studied by an introduction of the recombinant proteins into experimental animals or by testing activities in human plasma which contains either one or the other polymorph *ex vivo*. Also mice may be created that express one or other PCPB polymorph by the techniques of homologous recombination. These mice can thentested for their bleeding and fibrinolytic phenotype.

Distribution of PCPB Polymorphism in Individuals

The relative distribution of $PCPB_{Thr147}$ and $PCPB_{Ala147}$ polymorphs (DNA or protein) in a particular individual can be determined by identification of the different polymorphs of PCPB found within a human subject, most easily using a blood specimen. One could use any of a variety of techniques which would distinguish the two polymorphs from each other, based on any of the characteristics described above in which the forms were found to differ. Labeled antibodies specific to each protein polymorph can be used to identify the forms within a blood sample. Alternatively, DNA probes specific to the area within the PCPB DNA where the polymorphism occurs can be used to measure the presence of $PCPB_{Thr147}$ and $PCPB_{Ala147}$ within a patient sample and also to compare the relative distribution of the two species. In the preferred embodiment, PCR is used to amplify appropriate segments within genomic DNA isolated from a subject's blood, the amplified DNA is isolated and then identified using probes characertistic of the two polymorphs. This approach is described in Example 6.

Information concerning the relative distribution of PCPB polymorphs within the general population as compared with that found amongst a population known to be at risk forthrombotic disease can be accumulated by performing analysis of polymorph composition, as described

above, on a statistically significant number of human subjects chosen at random, as well as in a population of human subjects known to be at risk for thrombotic disease. Accumulation of sufficient data allows establishment of a PCPB polymorph profile for patients at risk ofthrombotic disease. With this profile established, one can screen individuals to identify those within the population at risk for thrombotic disease by determining a subject's PCPB polymorph profile and comparing the subject's values to the at-risk population profile. Such identication is useful in helping doctors to alert those individuals with a propensity toward thrombotic disease to the need for them to appropriately monitor their health and perhaps alter their behavior so as to reduce their risk.

* * * * *

The following specific preparations and examples are provided as a guide to assist in the practice of the invention, and are not intended as a limitation on the scope of the invention.

EXAMPLE 1: Isolation of cDNA coding for $PCPB_{Ala147}$ Polymorph

Complementary DNA coding for Ala_{147} polymorph of human plasma carboxypeptidase B (PCPB) was isolated from human liver Quick-Clone cDNA (Cat #7113-1, Promega, Madison, WI) by the polymerase chain reaction (PCR) method. The two primers used were 5'-GATGAAGCTTTGCAGCCTTGCA-3' (SEQ ID NO: 3) and 5'-CATTAAACATTCCTAATGACA-3' (SEQ ID NO: 4), based on the sequence published by Eaton *et al., J. Biol. Chem.* (1991), Vol 266, pp. 21833 - 21838. Liver cDNA (1 ng) was used as a template with Expand™ High Fidelity PCR kit (Cat #1732641, Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's specifications. The conditions for the Perkin Elmer Thermocycler consisted of 35 cycles of ;

denaturation at 94 °C for 1 minute
annealing at 50 °C for 45 seconds
elongation at 72 °C for 1 minute.

The 1.3 kb PCR fragment was subcloned into a pCR2.1 vector (Invitrogen, Carlsbad, CA) for DNA seuqencing. The DNA sequence was performed on both strands of the DNA using the dideoxy chain termination method described by Sanger *et al., Proc. Natl. Acad. Sci. USA.* 74: 5463 - 5467 (1977). The DNA sequence is shown in Figure 1. The DNA sequence isolated differed from the published sequence at two nucleotide positions, one at base 505 (A to G), which resulted in the substitution of threonine at residue 147 with alanine, and the other

change at base 678 (C to T), which led to a silent mutation.

EXAMPLE 2: Isolation of cDNA coding for $PCPB_{Thr147}$ Polymorph

Complementary DNA coding for Thr_{147} polymorph of human PCPB was isolated from total HepG 2 RNA by reverse transcriptase (RT)-PCR method. One µg of total HepG 2 RNA (from Dr. Q. Wu, Berlex Biosciences, Richmond, CA) was reverse transcribed using 40 unit of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim, Indianapolis, IN) in a 20 ul reaction mixture containing 50 mM Tris-HCl, 8 mM $MgCl_2$, 30 mM KCl, 1 mM dithiothreitol, pH 8.5, 0.2 µg of oligo (dT) primers, 5 mM each of dATP, dCTP, dTTP and dGTP, 20 unit of RNasin (Boehringer Mannheim) at 42 °C for 60 minutes. Following the heat inactivation at 65 °C for 10 minutes, 1 µl sample of HepG 2 cDNA was used as template in the PCR under the same conditions as described above. The PCR product was subcloned into the pCR2.1 vector, and the clone containing PCPB cDNA coding for Thr147 polymorph was identified by DNA sequencing.

EXAMPLE 3: Baculovirus Expression of human $PCPB_{Ala147}$ and $PCPB_{Thr147}$

The XHO1/KPN1 fragment of pCR2.1 vectors containing either $PCPB_{Ala147}$ or $PCPB_{Thr147}$ cDNA was subcloned into a pBacPAK 8 vector (Clontech, Palo Alto, CA) at XHO1/KPN1 sites in order to place the gene of interests under the control of the AcMNPV polyhedrin promoter. Baculovirus expression of recombinant PCPB polymorphs was performed by co-transfecting the plasmid pBacPAK8/PCPB with a linearized BacPAK6 viral cDNA (Clontech) into *Spodoptera frugiperda* (Sf9) cells according to the manufacturer's instructions. Recombinant plaques were identified and purified by their ß-galactosidase negative phenotype. Expression of PCPB protein was confirmed by Western blotting of the media harvested 3 days post infection, with monoclonal antibodies to PCPB purified from plasma (donated by Dr. L.Bajzar, Queen's University, Kingston, Ontario, Canada).

Sf9 cells expressing either $PCPB_{Ala147}$ or $PCPB_{Thr147}$ were grown as follows: Non-infected Sf 9 cells were grown in shake flasks at 28 °C, to a density of 1 - 1.2 x 10^6 per ml in TNMF (Grace's with supplements from Sigma, St. Louis, MO) plus 10 % FBS and 0.1 %pluronic F-68 (Sigma), and a viability of > 97 %. One liter of cells was infected with viral stock at MOI of around 0.01. Cultures were harvested between 48 and 72 h post-infection by centrifuging the media at 1200 rpm for 10 minutes. The media was then used for purification of both recombinant PCPB

proteins.

EXAMPLE 4: Purification of recombinant PCPB proteins

The conditioned media from Sf9 cells containing either recombinant $PCPB_{Ala147}$ or $PCPB_{Thr147}$ proteins were diluted 20-fold with QH_2O, the pH adjusted to 6.8, and millipore-filtered prior to S-Sepharose chromatography. A S-Sepharose column (Pharmacia Biotech Inc., Piscataway, NJ) was equilibrated with an equilibration buffer (20 mM phosphate buffer, pH 6.8). After applying the sample, the column was washed extensively with the equilibration buffer. Bound proteins were eluted from the column with a salt gradient of 0 to 0.5 MNaCl in the equilibration buffer. Ten-ml fractions were analyzed by SDS/PAGE electroporesis of denatured samples, followed by Western blotting using monoclonal antibodies to PCPB. Fractions containing recombinant PCPB were pooled and applied directly onto a plasminogen-affinity column. The plasminogen-affinity column had been prepared as follows; fourty one mg of plasminogen was purified from 650 ml of human plasma on a Lysine-Sepharose column (Pharmacia) according to the manufacturer's instructions. Plasminogen was dialysed against 0.1 M sodium citrate, pH 6.5, and was coupled at 4 °C overnight to 1.5 g of CNBr-activated Sepharose 4B (Pharmacia) that had been washed with 1 mM HCl just prior to use. The remaining active sites on the resin were blocked with 0.1 M Tris buffer, pH 8.0. The resin was washed three times alternatively with 0.1 M acetate buffer/0.5 M NaCl, pH 4.0 and 0.1 M Tris-HCl/0.5 M NaCl, pH 8.0, and finally equlibrated with phosphate-buffered saline (PBS) containing 1 µM D-Val-Phe-Lys chloromethyl ketone (VFL-CMK from Calbiochem-Novabiochem International, San Diego, CA). After applying samples, the column was washed extensively with PBS plus 1 µM VFL-CMK. Bound contaminants were eluted from the column with 5 mM epsilon-aminocaproic acid (ACA) in PBS. Recombinant PCPB was eluted with 200 mM epsilon ACA in PBS. Four ml fractions were collected into tubes, each containing 8 ml of 0.015 % Tween 80 in PBS. Fractions containing recombinant PCPB were identified using silver-staining after SDS/PAGE gel electrophoresis, pooled, and applied onto a small S-Sepharose column to remove epsilon ACA and to concentrate the sample, using essentially the same conditions as for the first column. The purity of the sample was determined by SDS/PAGE gel electrophoresis. The molecular weight of the two polymorphs of recombinant PCPB isolated in this manner were estimated to be around 50 k-dalton.

EXAMPLE 5: Activation and carboxypeptidase B activity assay of PCPB polymorphs

Recombinant PCPB (0.2 µM) was activated with 10 nM of thrombin (Sigma) and 50 nM of thrombomodulin (Solulin, Berlex Biosciences, Richmond, CA) in the activation buffer consisting of 20 mM HEPES, 0.15 M NaCl, 5.0 mM $CaCl_2$, pH 7.4, at room temperature for 10 minutes. Activation was stopped by an addition of 0.34 unit/ml hirudin (Sigma). Activation of recombinant PCPB was confirmed by the decrease in 50 K-dalton band and the appearance of 35 k-dalton fragment in SDS/PAGE gelelectrophoresis. Carboxypeptidase B-like activity of activated recombinant PCPB was measured by the hydrolysis of hippuryl-arginine (Sigma) to hippuric acid. In order to improve the sensitivity of assay, hippuric acid produced was converted to a chromogen with cyanuric chloride dissolved in dioxane, and absorbance of the chromogen was measured at 382 nm, according to the protocol described by Hendriks et al. in Clinica Chimica Acta: 157, 103 - 108 (1986). The assay has been adapted to a 96-well plate format as follows;

In a 96-well plate, add

24 µl HEPES (50 mM, pH 7.8)
12 µl QH_2O
12 µl activated PCPB (dilute 1:1 with the activation beffer)
12 µl hippuryl-Arg (10 mM in 20 mM NaOH)

Incubate at room temperature for 30 to 60 minutes.

To each well, add

80 µl phosphate buffer (0.2 M, pH 8.3)
60 µl cyanuric chloride (Sigma) in dioxane (3 %, w/v)

Mix well by pipetting several times and transfer clear supernatants to new wells, and read the absorbance at 382 nm (endpoint).

Include hippuric acid standard (starting with 2.5 mM stock solution, two-fold serial dilution in 20 mM NaOH).

24 µl HEPES (50 mM, pH 7.8)
12 µl QH_2O
12 µl the activation buffer
12 µl hippuric acid standards

EXAMPLE 6: Identification of PCPB polymorphism at residue 147 in blood specimens

In order to investigate the presence of polymorphism at residue 147 of PCPB in human subjects, PCPB DNA fragments were isolated from genomic DNA using the PCR method. Genomic DNAs were isolated from 200 µl of whole blood from various individuals using QIAamp Tissue Kit (QIAGEN Inc., Santa Clarita, CA). Up to 500 ng of genomic DNA was used as a template in the reaction mixture containing 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 0.2 mM each of dATP, dGTP, dCTP and dTTP, 50 pmole each of primers 5'-ATGGCCTATGAACCACAAG-3' (SEQ ID NO: 3) and 5'-GTTTCTGGAAAAGAACAA-3' (SEQ ID NO: 4). The conditions for the Perkin Elmer Thermocycler consisted 30 cycles of :

denaturation at 94 °C for 1 minute
annealing at 55 °C for 45 seconds
elongation at 72 °C for 1 minute.

Two plasmid DNAs, one coding for the $PCPB_{Ala147}$ polymorph and the other coding for the $PCPB_{Thr147}$ polymorph, were included as internal controls. The 105-base-long PCR products were run in 1.8 % agarose gel electrophoresis and transferred to a nylon membrane (Boehringer Mannheim) using 0.5 M NaOH/1.5 M NaCl. Two duplicate membranes were prepared, briefly washed in 2 X SSC (30 mM sodium citrate, pH 7.5, 0.3 M NaCl), UV cross-linked, and pre-hybridized in 5 X SSC, 1 % blocking reagent (Boehringer Mannheim), 0.1 % laurylsarcosine, and 0.02 % SDS for 20 minutes at 37 °C. The membranes were hybridized overnight at 37 °C in the pre-hybridization buffer containing digoxigenin (DIG)-labeled probes. Two oligonucletides, 5'-AAAGAACAAGCAGCCAAAA-3' (SEQ ID NO: 5) (corresponding to the sequence of $PCPB_{Ala147}$) and 5'-AAAGAACAAACAGCCAAAA-3' (SEQ ID NO: 6) (corresponding to the sequence of $PCPB_{Thr147}$), were labeled with DIG-11-ddUTP using terminal transferase (Genius 5 Oligonucleotide 3'-End Labeling kit from Boehringer Mannheim). The membranes were washed twice in 2 X SSC/0.1 % SDS at room temperature, followed by tetramethylammonium chloride (TMAC) wash solution, consisting of 3.0 M TMAC (Sigma), 50 mM Tris-HCl, pH 8.0, 0.1 % SDS and 2 mM EDTA. The membrane hybridized

with the DIG-Ala probe was washed twice, 30 minutes each, at 53 °C, while the membrane hybridized with the DIG-Thr probe was washed at 50 °C. DIG-labeled DNA fragments were detected using alkaline phosphatase conjugated anti-DIG antibodies and chemiluminescent substrate CSPD® (DIG/Genius™ 7 Luminescent Detection kit from Boehringer Mannheim). See Figure 4.

* * * * *

While the present invention has been described with reference to the specific embodiments thereof, it should be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the true spirit and scope of the invention. In addition, many modifications may be made and equivalents may be substituted without departing from the true spirit and scope of the invention. In addition, many modifications may be made to adapt a particular situation, material, composition of matter, process, process step or steps, to the objective, spirit and scope of the present invention. All such modifications are intended to be within the scope of the claims appended hereto.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: SCHERING AG, Berlin
Müllerstraße 178
D-13342 Berlin

(ii) TITLE OF INVENTION: Method of Detecting Thrombotic Disease Risk

(iii) NUMBER OF SEQUENCES: 6

(iv) CORRESPONDENCE ADDRESS:
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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/869,057
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(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 0049-30-468-12085
(B) TELEFAX: 0049-30-468-12058

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1272 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(x) PUBLICATION INFORMATION:
(A) AUTHORS: Eaton, Dan L.
Malloy, Beth E.
Tsai, Siao P
Henzel, William
Drayna, Dennis

(B) TITLE: Isolation, Molecular Cloning, and Partial Characterization of a Novel Carboxypeptidase B from Human Plasma
(C) JOURNAL: J. Biol. Chem.

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(E) ISSUE: 32
(F) PAGES: 21833-21838
(G) DATE: Nov 15-1991

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATGAAGCTTT GCAGCCTTGC AGTCCTTGTA CCCATTGTTC TCTTCTGTGA GCAGCATGTC 60

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GTTCTACAGA ATCTTACTAC AACATATGAG ATTGTTCTCT GGCAGCCGGT AACAGCTGAC 180

CTTATTGTGA AGAAAAAACA AGTCCATTTT TTTGTAAATG CATCTGATGT CGACAATGTG 240

AAAGCCCATT TAAATGTGAG CGGAATTCCA TGCAGTGTCT TGCTGGCAGA CGTGGAAGAT 300

CTTATTCAAC AGCAGATTTC CAACGACACA GTCAGCCCCC GAGCCTCCGC ATCGTACTAT 360

GAACAGTATC ACTCACTAAA TGAAATCTAT TCTTGGATAG AATTTATAAC TGAGAGGCAT 420

CCTGATATGC TTACAAAAAT CCACATTGGA TCCTCATTTG AGAAGTACCC ACTCTATGTT 480

TTAAAGGTTT CTGGAAAAGA ACAAACAGCC AAAAATGCCA TATGGATTGA CTGTGGAATC 540

CATGCCAGAG AATGGATCTC TCCTGCTTTC TGCTTGTGGT TCATAGGCCA TATAACTCAA 600

TTCTATGGGA TAATAGGGCA ATATACCAAT CTCCTGAGGC TTGTGGATTT CTATGTTATG 660

CCGGTGGTTA ATGTGGACGG TTATGACTAC TCATGGAAAA AGAATCGAAT GTGGAGAAAG 720

AACCGTTCTT TCTATGCGAA CAATCATTGC ATCGGAACAG ACCTGAATAG GAACTTTGCT 780

TCCAAACACT GGTGTGAGGA AGGTGCATCC AGTTCCTCAT GCTCGGAAAC CTACTGTGGA 840

CTTTATCCTG AGTCAGAACC AGAAGTGAAG GCAGTGGCTA GTTTCTTGAG AAGAAATATC 900

AACCAGATTA AAGCATACAT CAGCATGCAT TCATACTCCC AGCATATAGT GTTTCCATAT 960

TCCTATACAC GAAGTAAAAG CAAAGACCAT GAGGAACTGT CTCTAGTAGC CAGTGAAGCA 1020

GTTCGTGCTA TTGAGAAAAC TAGTAAAAAT ACCAGGTATA CACATGGCCA TGGCTCAGAA 1080

ACCTTATACC TAGCTCCTGG AGGTGGGGAC GATTGGATCT ATGATTTGGG CATCAAATAT 1140

TCGTTTACAA TTGAACTTCG AGATACGGGC ACATACGGAT TCTTGCTGCC GGAGCGTTAC 1200

ATCAAACCCA CCTGTAGAGA AGCTTTTGCC GCTGTCTCTA AAATAGCTTG GCATGTCATT 1260

AGGAATGTTT AA 1272

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 423 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(F) TISSUE TYPE: Plasma

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 23..401

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Pro Arg Thr Ser Arg Gln Val Gln Val Leu Gln Asn Leu Thr Thr Thr
        35                  40                  45

Tyr Glu Ile Val Leu Trp Gln Pro Val Thr Ala Asp Leu Ile Val Lys
    50                  55                  60

Lys Lys Gln Val His Phe Phe Val Asn Ala Ser Asp Val Asp Asn Val
65                  70                  75                  80

Lys Ala His Leu Asn Val Ser Gly Ile Pro Cys Ser Val Leu Leu Ala
                85                  90                  95

Asp Val Glu Asp Leu Ile Gln Gln Gln Ile Ser Asn Asp Thr Val Ser
            100                 105                 110

Pro Arg Ala Ser Ala Ser Tyr Tyr Glu Gln Tyr His Ser Leu Asn Glu
        115                 120                 125

Ile Tyr Ser Trp Ile Glu Phe Ile Thr Glu Arg His Pro Asp Met Leu
    130                 135                 140

Thr Lys Ile His Ile Gly Ser Ser Phe Glu Lys Tyr Pro Leu Tyr Val
145                 150                 155                 160

Leu Lys Val Ser Gly Lys Glu Gln Thr Ala Lys Asn Ala Ile Trp Ile
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Asp Cys Gly Ile His Ala Arg Glu Trp Ile Ser Pro Ala Phe Cys Leu
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Trp Phe Ile Gly His Ile Thr Gln Phe Tyr Gly Ile Ile Gly Gln Tyr
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Thr Asn Leu Leu Arg Leu Val Asp Phe Tyr Val Met Pro Val Val Asn
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Val Asp Gly Tyr Asp Tyr Ser Trp Lys Lys Asn Arg Met Trp Arg Lys
225                 230                 235                 240

Asn Arg Ser Phe Tyr Ala Asn Asn His Cys Ile Gly Thr Asp Leu Asn
                245                 250                 255

Arg Asn Phe Ala Ser Lys His Trp Cys Glu Glu Gly Ala Ser Ser Ser
            260                 265                 270

Ser Cys Ser Glu Thr Tyr Cys Gly Leu Tyr Pro Glu Ser Glu Pro Glu
        275                 280                 285

Val Lys Ala Val Ala Ser Phe Leu Arg Arg Asn Ile Asn Gln Ile Lys
    290                 295                 300

Ala Tyr Ile Ser Met His Ser Tyr Ser Gln His Ile Val Phe Pro Tyr
305                 310                 315                 320

Ser Tyr Thr Arg Ser Lys Ser Lys Asp His Glu Glu Leu Ser Leu Val
                325                 330                 335

Ala Ser Glu Ala Val Arg Ala Ile Glu Lys Thr Ser Lys Asn Thr Arg
            340                 345                 350

Tyr Thr His Gly His Gly Ser Glu Thr Leu Tyr Leu Ala Pro Gly Gly
        355                 360                 365

Gly Asp Asp Trp Ile Tyr Asp Leu Gly Ile Lys Tyr Ser Phe Thr Ile
    370                 375                 380

Glu Leu Arg Asp Thr Gly Thr Tyr Gly Phe Leu Leu Pro Glu Arg Tyr
385                 390                 395                 400

Ile Lys Pro Thr Cys Arg Glu Ala Phe Ala Ala Val Ser Lys Ile Ala
                405                 410                 415

Trp His Val Ile Arg Asn Val
            420
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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PRIMER"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATGGCCTATG AACCACAAG 19

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PRIMER"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GTTTCTGGAA AAGAACAA 18

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "OLIGONUCLEOTIDE"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AAAGAACAAG CAGCCAAAA 19

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "OLIGONUCLEOTIDE"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AAAGAACAAA CAGCCAAAA 19

WHAT IS CLAIMED IS:

1. A process for determining the presence of DNA or protein polymorphs of PCPB in a human subject, comprising:

obtaining a tissue or blood sample from the subject;

preparing the sample for analysis; and

determining the presence of PCPB polymorphs within the sample.

2. The process of Claim 1, wherein a blood sample is used.

3. The process of Claim 1, wherein the sample is prepared for analysis by isolation of the genomic DNA and amplification of the region of DNA coding for position 147 of the PCPB protein.

4. The process of Claim 3, wherein the presence of DNA coding for $PCPB_{Ala147}$ and/or $PCPB_{Thr147}$ is determined using DNA probes which hybridize selectively to genomic DNA coding for each of these molecules.

5. The process of Claim 4, wherein the DNA probes have the sequences 5'-AAAGAACAAGCAGCCAAAA-3' (SEQ ID NO: 5) and 5'-AAAGAACAAACAGCCAAAA-3' (SEQ ID NO: 6).

6. A process for determining the presence of DNA coding for the $PCPB_{Thr147}$ and/or $PCPB_{Ala147}$ polymorphs in a human subject, comprising

obtaining a blood sample from the subject;

isolating genomic DNA from the blood sample;

amplifying segments of the genomic DNA associated with the PCPB gene using PCR;

separating the products of PCR using gel electrophoresis;

immobilizing the gel-separated products by transfer to a nylon membrane;

contacting the membrane with PCPB -specific probes; and

measuring the amount of hybridization of the probes with the membrane-immobilized DNA.

7. The process of Claim 6, wherein hybridization is performed using DNA probes having the sequences 5'-AAAGAACAAGCAGCCAAAA-3' (SEQ ID NO: 5) and 5'-AAAGAACAAACAGCCAAAA-3' (SEQ ID NO: 6).

8. A process for determining the risk of thrombotic disease in a human subject, comprising obtaining a tissue or blood sample from the subject:
determining the presence of PCPB polymorphs within the sample by genetic orprotein analysis; and
comparing the subject's PCPB polymorph values with a PCPB polymorph profile for an at-risk population.

9. A process for determining the risk of thrombotic disease in a human subject, comprising obtaining a blood sample from the subject;
determining the presence of the genes coding for polymorphs $PCPB_{Thr147}$ and $PCPB_{Ala147}$ in the sample using a method comprising
obtaining a blood sample from the subject;
isolating genomic DNA from the blood sample;
amplifying segments of the genomic DNA associated with the PCPB gene using PCR;
separating the products of PCR using gel electrophoresis;
immobilizing the gel-separated products by transfer to a nylon membrane;
contacting the membrane with PCPB -specific probes; and
measuring the amount of hybridization of the probes with the membrane-immobilized DNA; and
comparing the values for that subject with an at-risk population profile.

10. The process of Claim 9, wherein hybridization is performed using DNA probes having the sequences 5'-AAAGAACAAGCAGCCAAAA-3' (SEQ ID NO: 5) and 5'-AAAGAACAAACAGCCAAAA-3 (SEQ ID NO: 6).

11. A test kit for identifying human subjects at risk forthrombotic disease, comprising DNA probes useful in measuring polymorphisms within the PCPB gene of the subject and a table useful for comparing the subject's PCPB polymorph values with those of an at-risk population.

12. A process for determining the presence of the $PCPB_{Thr147}$ and/or $PCPB_{Ala147}$ protein polymorphs in a human subject, comprising

obtaining a blood sample from the subject;

isolating protein material from the blood sample;

separating the proteins using gel electrophoresis;

immobilizing the gel-separated proteins by transfer to a nylon membrane;

contacting the membrane with antibodies specific to $PCPB_{Thr147}$ and $PCPB_{Ala147}$; and

measuring the amount of antibody bound to the membrane-immobilized proteins.

13. The process of Claim 11, wherein the antibodies are monoclonal.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 98/03244

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5 206 161 A (GENENTECH INC.,) 27 April 1993 cited in the application see the whole document ---	1-13
A	EATON D. L. ET AL.,: "Isolation, Molecular cloning, ad partial characterization of a novel carboxypeptidase B from human plasma" J. BIOLOGICAL CHEMISTRY, vol. 266, no. 32, - 15 November 1991 pages 21833-21838, XP002081105 cited in the application see the whole document --- -/--	1-13

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 October 1998	02/11/1998

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 98/03244

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category ˘	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	BAJZAR L. ET AL.,: "Purification and characterization of TAFI, a thrombin-activable fibronolysis inhibitor" J. BIOLOGICAL CHEMISTRY, vol. 270, no. 24, - 16 June 1995 pages 14477-14484, XP002081106 see the whole document ---	1-13
P,X	NAGASHIMA M. ET AL.,: "Identification and characterization of two TAFI isoforms" THROMBOSIS AND HAEMOSTASIS, vol. supp, - 6 June 1997 page 2432 XP002081207 see the whole document -----	1-13

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 98/03244

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5206161 A	27-04-1993	US 5364934 A	15-11-1994
		US 5474901 A	12-12-1995
		US 5593674 A	14-01-1997